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A novel high-content phenotypic screen to identify inhibitors of mitochondrial DNA maintenance in trypanosomes

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Kinetoplastid parasites cause diverse neglected diseases in humans and livestock, with an urgent need for new treatments. Survival of kinetoplastids depends on their uniquely structured mitochondrial genome (kDNA), the eponymous kinetoplast. Here we report development of a high-content screen for pharmacologically induced kDNA loss, based on specific staining of parasites and automated image analysis. As proof-of-concept we screened a diverse set of ~14,000 small molecules and exemplify a validated hit as a novel kDNA-targeting compound.

21 **Keywords:** High-throughput screening, high-content screening, trypanosomatids, kinetoplast,
22 kDNA, mitochondria

23

24 **Introduction, methods, results and discussion combined**

25 Kinetoplastids cause diverse, life-threatening diseases in humans and their livestock,
26 namely African sleeping sickness (1), Chagas disease (2) and the leishmaniasis (3) in the former
27 and animal trypanosomiasis in the latter (4). These diseases particularly affect populations in
28 low- and middle-income countries in many parts of the world. Currently available drugs are
29 unsatisfactory because they cause severe, and sometimes lethal, side-effects, they are difficult to
30 administer, and resistance continues to emerge, necessitating the development of novel anti-
31 kinetoplastid therapies (5, 6).

32 Although kinetoplastids have evolved distinct methods of infection and host immune
33 evasion, they all share a unique biological feature: the organisation of their mitochondrial DNA
34 (mtDNA, or kDNA in these organisms) in a peculiar structure that gave these organisms their
35 name: the kinetoplast (7). The kDNA is extremely complex, containing hundreds of different
36 classes of 'guide RNA'-encoding minicircles of variable copy number which are essential for
37 post-transcriptional RNA editing in these organisms (8–10). Together with dozens of
38 maxicircles, which are the equivalent of mtDNA in other eukaryotes and encode subunits of the
39 respiratory chain, F₁F₀-ATP synthase and mitoribosomes, thousands of minicircles form an
40 interlinked network structure. The kDNA is thus intrinsically different from mammalian mtDNA,
41 is essential for parasite survival (11, 12) and is a validated target for some current anti-

trypanosomatid therapies (13–16), making it an attractive target for discovery of new, improved drugs (17, 18).

Uniquely among kinetoplastids, the sole function of kDNA in bloodstream form *T. brucei* is the production of subunit *a* of the F_1F_0 -ATPase (19), which in this stage of the parasite's life cycle operates in reverse to maintain the mitochondrial membrane potential (20). The respiratory chain and oxidative phosphorylation - classical mitochondrial functions - are not functional in bloodstream stage *T. brucei*. Facilitated by this limited function, kDNA-independent mutants have evolved in *T. brucei* subspecies that cause trypanosomiasis in animals (19, 21, 22). Typically, kDNA independence in *T. brucei* is caused by a mutation in the nuclearly encoded subunit γ of the mitochondrial F_1F_0 -ATPase (19). Importantly, kDNA independence has never been reported for those kinetoplastid parasites of humans and livestock that are currently responsible for by far the greatest disease and economic burden, i.e. *Leishmania* spp., *T. cruzi*, *T. vivax* and *T. congolense*. This remains to be the case despite decades of use of ethidium bromide (EtBr) and isometamidium chloride (phenanthridine compounds that strongly affect kDNA) for the treatment of African animal trypanosomiasis (14–16, 23–25). Loss of kDNA can apparently not be compensated for in these species, either because additional kDNA-encoded genes are essential (clearly the case for *Leishmania* and *T. cruzi*, which depend on a functional respiratory chain throughout their life cycle (26)), or because the mutations in F_1F_0 -ATPase γ that can compensate for loss of kDNA in bloodstream *T. brucei* are not functional in these species. Novel anti-trypanosomatid therapies based on inhibition of kDNA maintenance are therefore attractive (17, 18).

Drug discovery efforts are typically either phenotypic or target-based (27, 28). While target-based campaigns have dominated efforts for decades, they often fail to produce new

therapeutic molecules due to the challenge of translating promising results from reductionist biochemical and cellular assays into robust efficacy in more complex *in vivo* models (29). In contrast, phenotypic screens are often more time-consuming and expensive, and the mode(s) of action behind any identified hits are usually unknown (29). However, both approaches are complimentary and can be used synergistically to fast-track the identification of target-specific compounds that can enter the cell and reach the associated intracellular organelles to induce the desired effect. This paper describes the design, implementation and validation of a phenotypic high-content screen (HCS) with automated image analysis for the discovery of hit compounds that specifically target kDNA maintenance, using *Trypanosoma brucei brucei* (hereafter referred to as *T. brucei*), a causative agent of animal trypanosomiasis, as a model system.

HTS design and image analysis. To enable the discovery of target-specific compounds, our phenotypic screen uses a genetically engineered kDNA-independent bloodstream form *T. brucei* cell line which tolerates kDNA loss due to an L262P mutation in the nuclearly encoded subunit γ of the mitochondrial F_1F_0 -ATPase (19). Non-specific cytotoxic or cytostatic compounds, or more general inhibitors of mitochondrial function, which would be more likely to cause side effects in the host, can readily be identified in this genetic background.

Our HCS has been optimized for use in a high throughput 384-well format (V-bottom, Greiner-Bio, #781280), using a Biomek FX liquid handler (Beckman) to dilute all compounds and subsequently adding L262P *T. brucei* cells using a VIAFLO multi-well plate liquid handler (Integra) in a class II biosafety cabinet. Briefly, 2.5 μ l compound (at a concentration of 200 μ M in culture medium with 2% dimethyl sulfoxide (DMSO)) were added to each well. Subsequently, 47.5 μ l of parasite culture in complete HMI-9 medium (30), supplemented with 20% (v/v) fetal calf serum, were seeded at 50 cells per well, giving a total volume of 50 μ l with 1×10^3 cells/ml

88 and a final compound concentration of 10 μ M. Plates were incubated in an atmosphere of 5%
89 CO₂ at 37°C for 4 days (31). Following incubation, cells were stained with the cytoplasmic
90 viability stain, 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; CAS: 150347-
91 59-4) at 10 μ M for 15 mins at 37°C and, consecutively and without any washing steps, with
92 Hoechst 33342 nucleic acid stain at 1 μ g/mL for 5 minutes at 37°C. Subsequently, cells were
93 fixed with 2% (w/v; final concentration) formaldehyde, with vigorous mixing to avoid clumped
94 cells, a step that is crucial for subsequent image analysis (Fig. 1A). After 24 h fixation at 4°C,
95 cells were washed 3 times with phosphate-buffered saline by centrifuging plates at 1,000 x g for
96 1 min to remove any remaining dye. Loss of cells during washing steps was minimised by using
97 V-bottom plates and carefully adjusting fixed pipette positions for the Biomek FX liquid handler.
98 Cells were then transferred into 384-well F-bottom plates for imaging (Greiner-Bio, #781986).
99 The plates were centrifuged at 1,000 x g for 5 min prior to imaging acquisition at 40x
100 magnification using an automated ImageXpress-XLS micro (Molecular Devices) HCS system.
101 Each well was imaged across four different fields of view using DAPI (for Hoechst 33342 stain)
102 and FITC (for CFDA-SE) filter sets. Image analysis was performed using the CellProfiler 3.1.9
103 software (32). Briefly, nuclear DNA and kDNA were identified based on area size of Hoechst
104 33342 positive objects, and viable cells were identified using the FITC channel (Fig. 1B, Fig.
105 S1).

106 **HCS performance validation and pilot screen.** Plates (n=2) were prepared as above,
107 with even-numbered columns containing a negative control (0.1% DMSO) and odd-numbered
108 columns containing 10 nM EtBr (in 0.1% (v/v) DMSO), a known inhibitor of kDNA
109 maintenance, as a positive control (15). A 'robust' Z' assay performance score of 0.725 was
110 calculated (33, 34), indicating excellent performance (35).

111 To test the ability of our HCS to identify novel inhibitors of kDNA maintenance, 13,486
112 compounds were screened, from a diverse set of chemical libraries: Prestwick Chemical Library
113 (Prestwick Chemical; 1,280 compounds), Screen-Well PKE library (Enzo Biochem; consisting
114 of protease (53), kinase (80) and epigenetic (43) inhibitors), and BioAscent 12K diverse
115 chemical libraries (BioAscent Discovery Ltd; 11,970 compounds). The Prestwick Chemical
116 library was designed to represent broad pharmacological diversity of all FDA-approved small
117 molecule drug classes and consists of drugs with known pharmacology, toxicology and
118 pharmacokinetic properties to support repurposing of existing drugs. The BioAscent 12K
119 compound library is a subset representing the chemical diversity of a 125,000-compound parent
120 library, enabling subsequent expansion of screening hits to explore structure-activity
121 relationships. All compounds were screened at a final concentration of 10 μ M in '0.1% (v/v)
122 DMSO in a 384-well format, where the first four columns had alternating positive (EtBr) and
123 negative (DMSO) controls. Additionally, the PKE and Prestwick Chemical libraries were also
124 screened at a lower final concentration of 1 μ M because both libraries have been reported to lead
125 to the identification of potent inhibitors in different phenotypic screening assays at this lower
126 dose which may better reflect on-target rather than off-target activity observed at higher doses
127 (36, 37). The screens were performed in 5 batches (48 plates in total), with a 'robust' Z' assay
128 performance score (34) ranging from 0.63 to 0.9 between batches. The HCS identified 152
129 compounds with a reduced ratio of kDNA per nucleus (Z-score < -2; Fig. 2 and Table S1).
130 Separate results for nucleus and kDNA counts for all wells are shown in Fig. S2.

131 **Hit validation.** For the top 50 compounds, based on ranking by kDNA/nucleus ratio
132 (excluding all compounds that had less than 50 DNA objects per well) and a Z-score < -2 (Table
133 S1), we manually reviewed the microscopy images for evidence of complete kDNA loss. Ten

134 candidates (Table S1) were cherry-picked for follow-up analysis based on consistently observed
135 loss of kDNA from cells treated with these compounds and on their commercial availability.
136 Purchased compounds were dissolved in DMSO, and their potency against wild type (WT) *T.*
137 *brucei* cells was evaluated using an adapted 3-day Alamar Blue method (19). Only two
138 compounds, (S)-propranolol hydrochloride and 1-(1-Adamantyl)-4-[(2-methoxy-4,5-
139 dimethylphenyl)sulfonyl]piperazine (AMDSP, BioAscent code BCC0052412) were sufficiently
140 potent at the highest concentration that could be tested (due to limited solubility in water) to
141 permit calculation of IC₅₀ values for WT cells of 16-22 μ M and 1.6-2.3 μ M, respectively (95%
142 confidence intervals, Table S1; the other 8 compounds did not significantly effect growth of WT
143 cells in the Alamar Blue assay). Next, we assessed the specificity of these two compounds as
144 inhibitor of kDNA maintenance. This specificity is indicated by the selectivity for killing of
145 kDNA-dependent ('WT') and kDNA-independent ('L262P'), but otherwise isogenic *T. brucei*
146 cells. The most specific compound reported to date is EtBr, with a selectivity index of ~300 in
147 the modified Alamar Blue assay (38). One the two compounds tested, AMDSP (Fig. 3A),
148 reproducibly affected the viability of WT *T. brucei* cells at a lower concentration compared to
149 L262P cells (Fig. 3B). The IC₅₀ for WT cells was 1.9 μ M, while the IC₅₀ for L262P cells was
150 estimated to be in the range of 8 μ M (the value could not be determined more precisely due to
151 poor compound solubility in DMSO at higher than 12.5 mM stock concentration). To investigate
152 the time required for AMDSP to affect growth, we performed growth curves in WT and L262P
153 cells at a final compound concentration of 12.5 μ M in 0.1% (v/v) DMSO (Fig. 3C and 3D). After
154 3 days of AMDSP treatment, growth of WT cells was much more severely inhibited compared to
155 L262P cells. No growth was observed between days 3 and 4 for one of the WT replicates (Fig.
156 3C, open red circles). The cumulative growth curve for the other replicate indicated a slight

157 increase in cell numbers between days 3 and 4 (Fig. 3C, filled red circles). However, by
158 microscopy, we found no intact and motile WT cells after 4 days for either WT replicate, even
159 after concentration of the culture by centrifugation, while L262P cells survived. Hence, it is more
160 likely that the apparent increase for one of the WT replicates was caused by counting of cell
161 debris in the Coulter machine. Moreover, we observed a substantial increase in the proportion of
162 cells with complete loss of kDNA (0K1N cells) in WT and L262P cells after 2 or 3 days of
163 exposure to 12.5 μ M AMDSP (Fig. 4A). Interestingly, loss of kDNA was more severe for WT
164 cells than for L262P cells. This could suggest a reduced uptake of AMDSP in L262P cells,
165 perhaps caused by the lower mitochondrial membrane potential in these cells (39). In further
166 support of an effect of AMDSP on kDNA maintenance, for the proportion of WT cells that had
167 retained at least some kDNA after AMDSP-treatment, we observed a significant kDNA reduction
168 in size compared to control cells (Fig. 4B), while the size of the nucleus was not affected (Fig.
169 S3).

170 Altogether, these data confirm that an important part of the mode of action of AMDSP in
171 trypanosomes is interference with kDNA maintenance. The data are consistent with the dynamics
172 of growth inhibition and effects on kDNA of other compounds that preferentially target this
173 structure, such as EtBr (39–41), although, unsurprisingly, potency and selectivity of this primary
174 hit are much lower. Nonetheless, AMDSP may represent a promising starting point for hit-to-
175 lead development. The compound is composed of piperazine, benzene and adamantane rings
176 with a tertiary sulfonamide group. Adamantane derivatives, such as the well-studied drug,
177 amantadine (1-amino-adamantane), show good pharmacokinetics in humans, are licensed drugs
178 for the treatment of Parkinson's disease, and in the past had been used for the treatment of
179 influenza, until emergence of resistance halted its application for this purpose (42). Moreover,

180 the discovery of amino-adamantane derivatives with trypanocidal activity (43) has spurred
181 efforts for the recent development of more potent adamantane-benzene derivatives (44).
182 Piperazine-based anti-helminthic drugs (45) have also gained interest in drug design studies
183 because of their trypanocidal activity (46). The exact mechanism(s) by which the described
184 derivatives affect trypanosomatids remains unknown but, based on our findings, effects on
185 kDNA should be explored. Furthermore, similarity searches with AMDSP of the full BioAscent
186 library suggest up to 150 related compounds that could be tested against trypanosomatids in the
187 future to explore structure-activity relationships.

188 **Identification of other anti-trypanosomatid compounds with unknown mode of**
189 **action.** In addition to a novel inhibitor of kDNA maintenance, we also identified compounds that
190 strongly affected the viability of the kDNA-independent *T. brucei* cell line used for screening
191 and that therefore must act via a different mechanism. To find such trypanocidal or trypanostatic
192 hits, we first corrected for positional growth effects in our plates using the median polish
193 normalisation method (47, 48) (Fig. S4). Median polish normalisation was performed in Spotfire
194 software (PerkinElmer) using the High Content Profiler package to remove row and column
195 biases. This method uses the row and column medians to identify the row and column effect on
196 the data. We then scored for hits affecting *T. brucei* viability based on less than 10 total nuclei
197 per image with Z-scores < -1. We identified 337 hits, corresponding to a hit rate of 2.5% (Table
198 S2; Fig. S5, left panel). These include 31 compounds from the Prestwick Chemical Library that
199 inhibited trypanosome growth at both 10 μ M and 1 μ M (double underline in Table S2),
200 suggesting a good starting potency for any lead development efforts. Incidentally, among the
201 compounds tested in our proof-of-concept screen were 9 compounds with known anti-
202 trypanosomatid activity (49). Seven of these compounds were among the hits with a Z-score < -1

(highlighted in Table S2, right panel in Fig. S5). This further confirms the robustness of our HCS assay and suggests that, as an additional benefit, the outputs from this assay could also be used for the identification of anti-trypanosomatid compounds with a mode-of-action unrelated to kDNA maintenance.

In conclusion, we successfully established and validated a scalable, kDNA maintenance based phenotypic HCS with automated image analysis, using an engineered kDNA-independent *T. brucei* cell line as a kinetoplastid model system. A proof-of-concept screen of diverse small compound libraries identified and validated a novel compound affecting kDNA maintenance in *T. brucei*. To the best of our knowledge, this is the first HCS specifically designed to identify inhibitors of kDNA maintenance. Furthermore, we identified other anti-trypanosomatid compounds with activity in the low micromolar range (but with unknown molecular targets) that could be useful starting points for trypanosomatid drug development. In the future, the screen could be further optimised by trying to address the positional growth effects in plates and by developing machine learning algorithms that can lower the rate of false-positive hits and detect more subtle changes in kDNA, nuclear DNA and cell morphology.

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369

370 **Figure legends**

371

372 **FIG 1. High-content screening (HCS) strategy to identify compounds inhibiting kDNA**
373 **maintenance in *T. brucei*.** (A) Representative fluorescence microscopy images of *T. brucei*
374 using the HCS staining protocol. From left to right: Hoechst 33342 staining of trypanosome
375 nuclei and kDNA (in magenta), CFDA-SE cytoplasmic viability stain (in green), phase contrast,

376 and merged images. **(B)** Schematic representation of the image analysis pipeline using
377 CellProfiler. First, nuclei and kDNA were identified from the Hoechst 33342 staining (upper left
378 panel). Next, nuclei and kDNA were separated by classifying stained objects according to area
379 size (upper right and lower right panels; nuclei ≥ 60 area size in arbitrary units, green in lower
380 right panel; kDNA < 60 area size, magenta in lower right panel; bin width = 20 with bin centre
381 ranging from 0 to 200). Finally, viable cells were identified using the CFDA-SE cytoplasmic
382 viability stain (lower left panel). Each well was imaged at four different, non-overlapping
383 positions.

384

385 **FIG 2. HCS result and hit selection.** Tested compounds were ranked based on the decrease of
386 kDNA/nucleus ratio in imaged wells (Z-score < -2 (dashed black line)), resulting in 152 hits (see
387 also Table S1). Images of the top 50 hits (based on ranking by decrease in kDNA/nucleus ratio)
388 were then re-examined using ImageJ software. Ten compounds were selected for follow-up
389 analysis, based on complete loss of kDNA observed and on commercial availability (highlighted
390 by the black triangles).

391

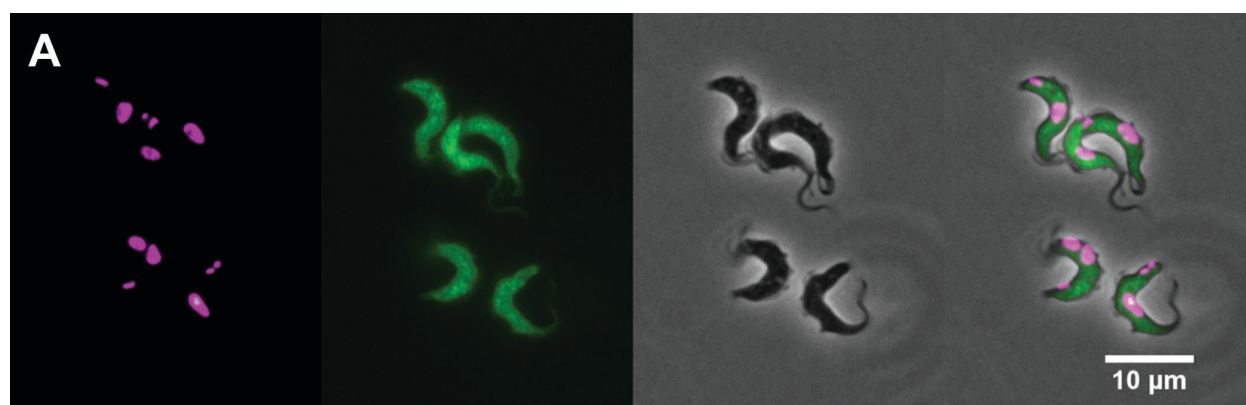
392 **FIG 3. Hit validation.** **(A)** Structure of AMDSP (BCC0052412). **(B)** Dose-response curves for
393 the effect of AMDSP on growth of kDNA-dependent (WT, black squares) and kDNA-
394 independent (L262P, red squares) bloodstream form *T. brucei*. **(C)** Cumulative growth curves of
395 bloodstream form *T. brucei* cells cultured in the presence (dashed lines) and absence (solid lines,
396 filled circles) of 12.5 μ M AMDSP (red) or 10 nM EtBr (blue). Growth curves in the presence of
397 solvent only are shown as controls (0.1% DMSO, black). Cell numbers were determined with a

398 Coulter counter. **(D)** Comparison of cumulative cell numbers in **(C)** after 96 h between WT and
399 L262P cells. Student unpaired t-test, $p < 0.00005$ (****). All experiments were performed in
400 triplicate; in addition, the effect of AMDSP on WT and L262P cells was tested on two separate
401 occasions (Test 1 and Test 2).

402

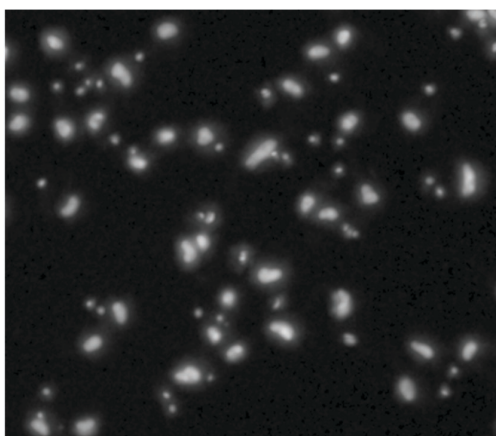
403 **FIG 4. AMDSP effects kDNA maintenance.** **(A)** Loss of kDNA (0K1N cells = cells with no
404 kinetoplast and one nucleus) assessed by DAPI staining and microscopy after 2 days (D2) and 3
405 days (D3) of culturing in the presence or absence of 12.5 μM AMDSP. Statistical significance of
406 differences was assessed with the Student unpaired t-test; $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$
407 (***). **(B)** The relative amount of kDNA in 1K1N cells (cells with 1 kinetoplast and 1 nucleus)
408 after 2 days of culturing was assessed by DAPI staining and quantitation of kinetoplast versus
409 nucleus fluorescence intensity. Statistical significance of differences was assessed with the
410 Mann-Whitney test; $P < 0.001$ (***) for AMDSP at 12.5 μM in 0.1% DMSO ($n = 90$) versus
411 0.1% DMSO ($n = 90$); $P < 0.001$ (***). All experiments were performed in triplicate.

412

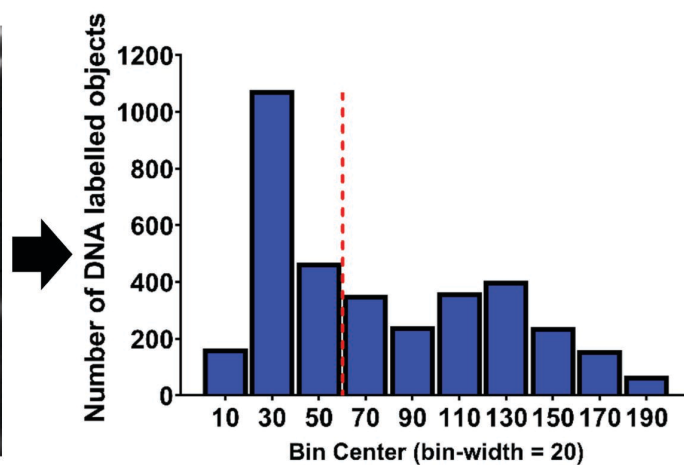


B

Identify all DNA labelled objects
(Hoechst stain)



Split DNA labelling into nuclear and kDNA
objects based on area size



Identify viable cells
(CFDA-SE stain)

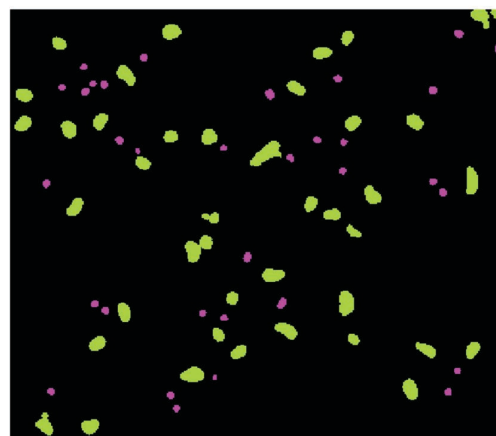
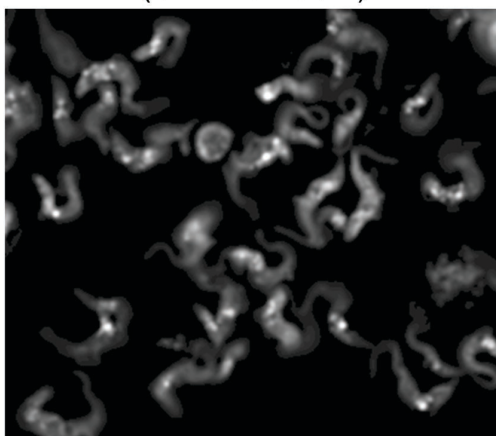
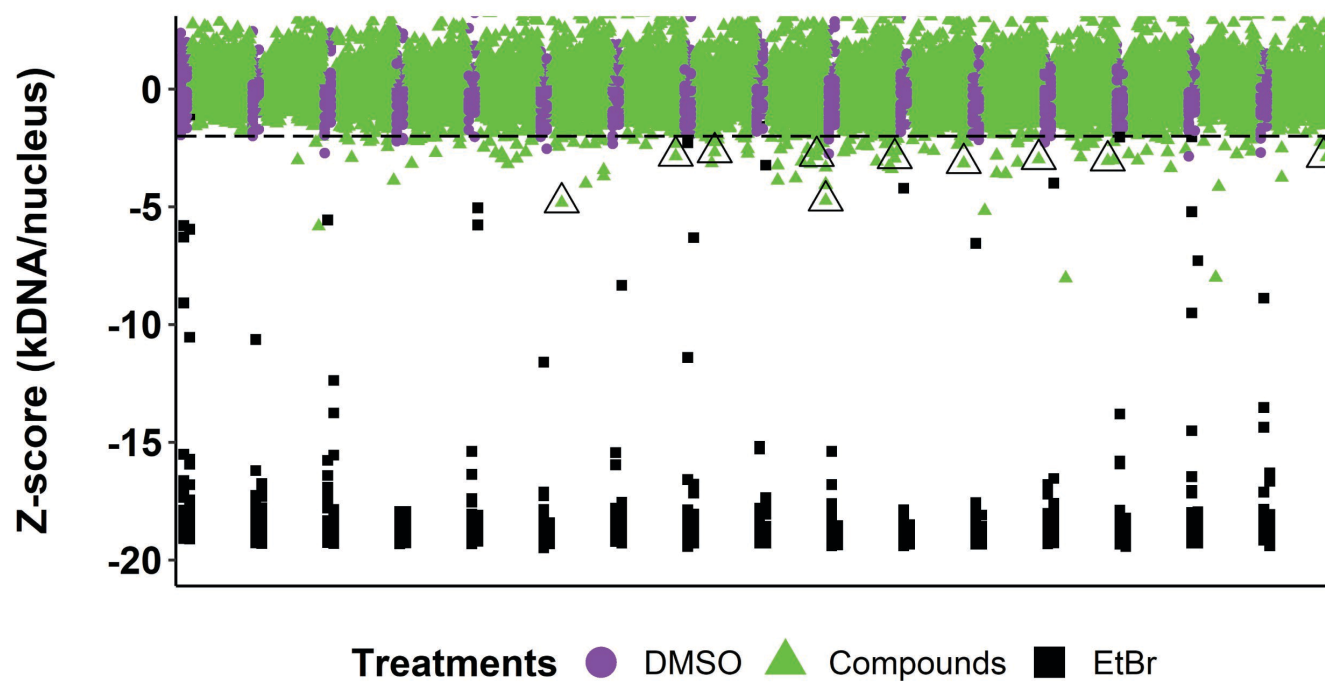


FIG 1

**FIG 2**

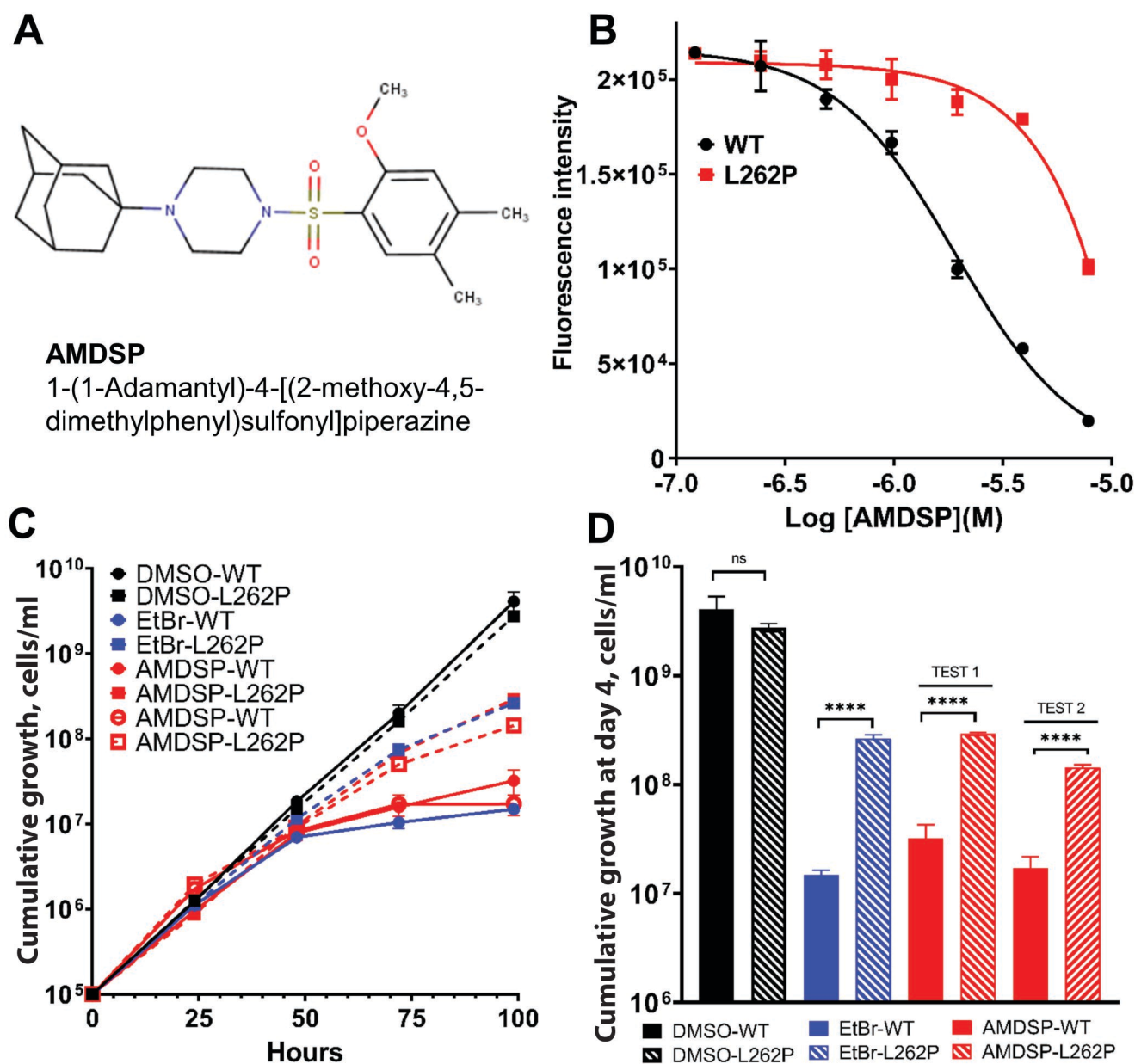


FIG 3

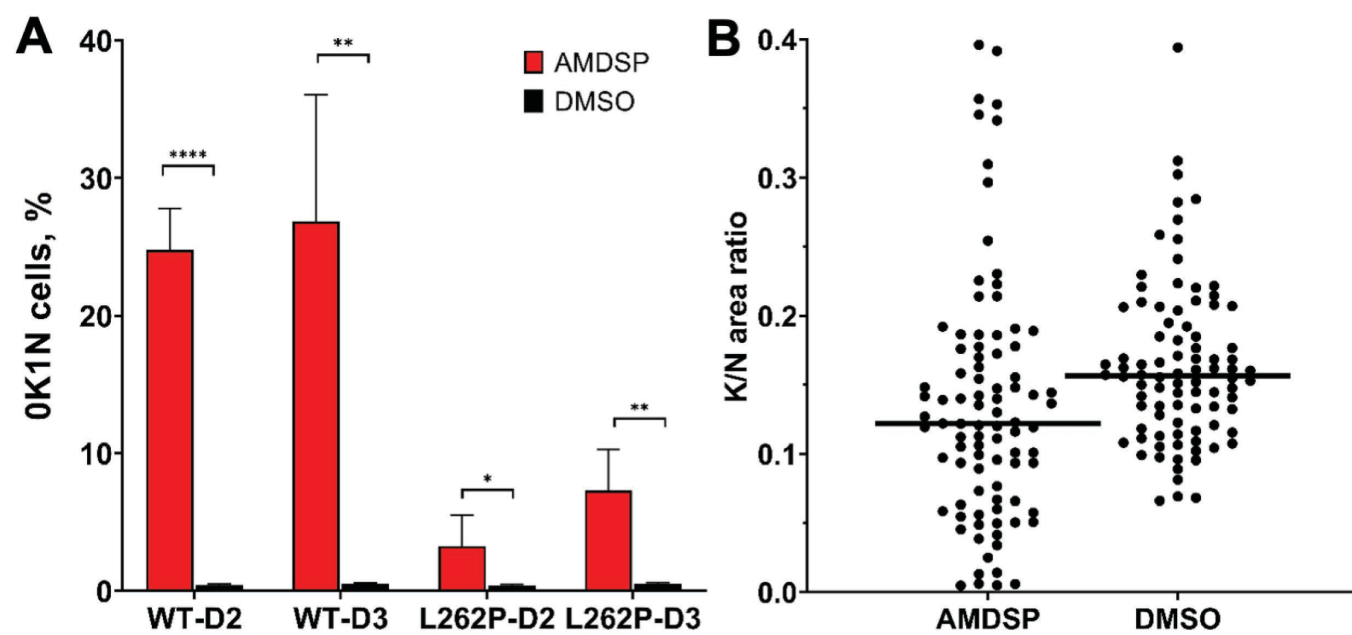


FIG 4